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Polymer-Encased Nanoparticles

Stabilization of Virus-like Particles with Poly(2-oxazoline)s**

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Since the introduction of virus-like particles (VLPs) as nanoscale building blocks,[1] they have become a favored material to fill the gap between supramolecular chemistry and microfabricated systems for medicine, materials science, and biotechnology. One great advantage of VLPs is their precisely defined structures, forming capsules for the packaging of proteins, catalysts, small molecules, and other entities,[2] as well as for the display of functional molecules on their outer surfaces.[3] The stabilities of chemically modified particles are often approximately the same as the underivatized scaffolds, but in some cases stability is decreased.[4]

Viruses and virus-like particles are often more resistant to denaturation and proteolytic cleavage than other proteins. Additional stability is sometimes accomplished in nature by the formation of covalent connections between individual capsid subunits.[5] Certain mimics of this strategy have proven successful,[4d] but others have not.[4c,e] We describe here an alternative approach in which a capsid surface is covered by polymer chains to which multiple connections are made, thereby cross-linking protein cage subunits. Polymers have most often been attached at one end to viruses and virus-like particles for the purpose of extending in vivo circulation lifetime, diminishing nonspecific adsorption, or passivating the immune response (Figure 1 a).[4b,6] Recent attention has been paid to the entrainment or growth of polymers inside protein nanoparticles (Figure 1 b),[4h,7] and the polymerization of shells around nanoparticles for the purpose of materials synthesis (Figure 1 c).[8] We describe here the first example of attachment of polymer chains to protein cages by discrete covalent contacts at multiple points (Figure 1 d), similar to the stabilization of liposomes by a polymer net.[9] An elegant report by Douglas and co-workers describes the construction of anchored cross-linked polymeric materials inside such a particle.[10]

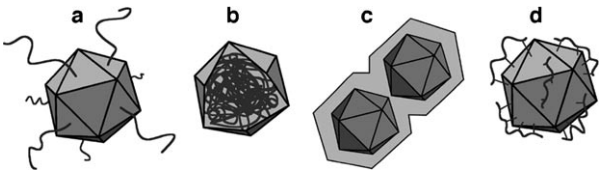

Figure 1. Various ways in which polymers have been combined with protein nanoparticles.

Poly(2-oxazoline)s have advantageous properties of versatile controlled syntheses by means of living cationic polymerization, aqueous-phase solubility, and chemical stability that make them attractive for a variety of biomedical and materials applications.[11] Several years ago we pioneered their derivatization with copper-catalyzed azide–alkyne cycloaddition (CuAAC) click chemistry,[12] for which they are very well suited.[13] By incorporating several "clickable" functional groups in the polymer chain, we hoped that virus-based hybrid structures could be constructed by cross-linking capsid coat proteins with poly(2-oxazoline) (POx) chains.

For the protein component we chose the icosahedral VLP formed from 180 copies of the coat protein of bacteriophage Qβ expressed recombinantly in *E. coli*.[14] The 132 amino acid subunit forms a noncovalently interlocked dimer; each subunit has four exposed amino groups on its outer surface that are accessible for covalent modification. The particle is further stabilized by intra- and inter-subunit disulfide bonds located at the five- and sixfold axes of symmetry, respectively.[4d] For the experiments described here, the standard Qβ VLP was derivatized with azido-*N*-hydroxysuccinimide ester reagent **1** at a concentration previously observed to acylate almost all of the 720 amino groups available on the exterior surface of the capsid (Figure 2 a).[15]

A series of complementary POx polymers was designed to allow comparison of single- and multiple-point attachment methods (Figure 2). The monomers 2-methyl-2-oxazoline (MeOx) and 2-ethyl-2-oxazoline (EtOx) were used along with 2-(pent-4-ynyl)-2-oxazoline (PynOx) to provide alkyne linkage points on a hydrophilic polymer.[13a] Propargyl-$MeOx_{60}$ (**P1**) and propargyl-$EtOx_{60}$ (**P2**) were prepared with a single alkyne at the terminus, whereas P($EtOx_{20}$-$PynOx_{2.5}$) (**P3**) and P($MeOx_{45}$-$PynOx_5$) (**P4**) incorporated alkynes

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Supporting information for this article (complete experimental details, including the preparation and characterization of VLPs, polymers, and conjugates) is available on the WWW under http://dx.doi.org/10.1002/anie.201006134.

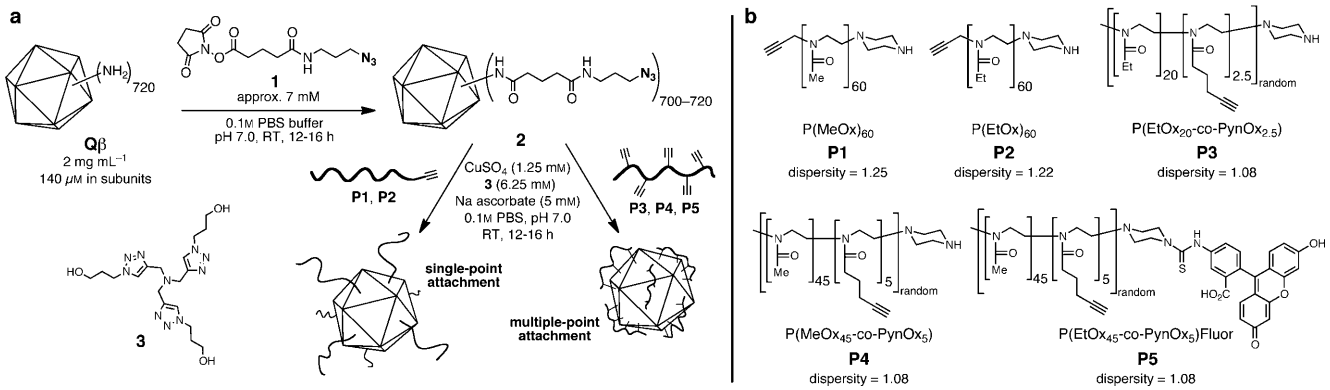

Figure 2. a) Preparation of POx-coated Qβ virus-like particles. Two general architectures of core–shell nanoparticles are produced, deriving from polymers with a single attachment point or from polymers with multiple attachment points. PBS = phosphate-buffered saline. b) Telechelic (**P1**, **P2**) and random copolymer (**P3**–**P5**) poly(2-oxazoline)s with terminal and pendant alkyne groups, respectively.

randomly in approximately 10% of the polymer pendant groups. All polymers carried a terminal amine moiety to allow for additional modification with a functional molecule such as a fluorescent dye or targeting moiety. As expected for cationic ring-opening polymerization,[16] the polymers were well-defined with low dispersities ($M_w/M_n \leq 1.25$). All polymers were fully characterized by ^{1}H NMR spectroscopy, GPC, and MALDI-TOF mass spectrometry (Supporting Information).

Using a fluorogenic coumarin azide,[17] we optimized the conditions for CuAAC bioconjugation to the polymeric alkyne POx using accelerating ligand **3**, as described in the Supporting Information. It was necessary to increase the concentrations of Cu and ligand fivefold over that normally used for bioconjugation[12c] to achieve maximal rates, perhaps because of competitive binding of Cu ions by the POx chains. End-labeled **P5** was also prepared to quantify polymer attachment to Qβ particles by virtue of fluorescein UV/Vis absorption, but the dye was found to induce particle aggregation (see the Supporting Information).

Size-exclusion chromatography (SEC) showed a progressive decrease in elution volume for the Qβ-**P4** particles with the use of increasing concentrations of **P4** in the CuAAC reaction (Figure 3a), indicating a gradual increase in hydrodynamic radii of polymer-coated capsids by virtue of the attachment of greater numbers of polymer chains. By this measure, maximum polymer loading was achieved in reactions using between 400 and 800 equivalents of POx per particle. This was corroborated by dynamic light scattering (DLS), showing a similar increase of the hydrodynamic diameter, to a maximum of approximately 38 nm, with increasing concentration of the POx reagent in the attachment reaction (Figure 3b). This value represents the addition of a 5 nm thick polymer shell to the particle surface, since native Qβ has a diameter of approximately 27 nm. Similar data were obtained for Qβ-**P1**, Qβ-**P2**, and Qβ-**P3** conjugates (Table 1 and the Supporting Information), showing that particle size can be controlled by the nature and amount of polymers grafted to the surface by click conjugation. The polymer-coated nanoparticles also appeared as well-formed icosahedra by transmission electron microscopy (TEM), as shown in Figures 3d–i and the Supporting Information.

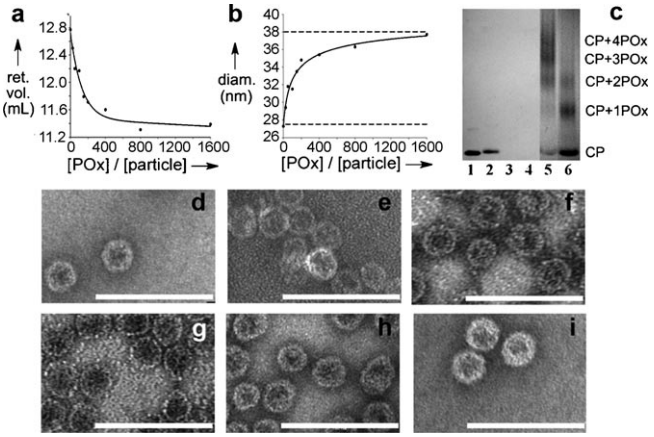

Figure 3. a) Retention volume shift in SEC of the products derived from increasing amounts of **P4** in the coating reaction (25 to 1600 equiv with respect to Qβ particles). b) Hydrodynamic diameter increase of the Qβ-POx (**P4**) conjugates, as measured by dynamic light scattering. c) Denaturing polyacrylamide gel electrophoresis of purified conjugates. Lanes: 1 = starting Qβ VLPs, 2 = Qβ-N_3 (**2**), 3 = Qβ-**P3**$_{298}$, 4 = Qβ-**P4**$_{144}$, 5 = Qβ-**P1**$_{244}$, 6 = Qβ-**P2**$_{146}$. d–i) TEM images (magnification 245000×) of VLPs; scale bars 100 nm: d) wild-type Qβ, e) Qβ azide **2**, f) Qβ-**P1**$_{244}$, g) Qβ-**P2**$_{146}$, h) Qβ-**P3**$_{298}$, i) Qβ-**P4**$_{144}$.

Table 1: Summary of the analysis of the Qβ-POx core–shell particles.

Construct	Diameter [nm][a]	ΔSEC [mL][b]	Alkynes/POx	Chains/particle[c]
Qβ-**P1**$_{244}$	43.6	2.1	1	244
Qβ-**P2**$_{146}$	35.6	1.7	1	146
Qβ-**P3**$_{298}$	35.8	1.0	2.5	298
Qβ-**P4**$_{144}$	37.6	1.4	5	144

[a] Measured by dynamic light scattering. [b] Change in retention volume on Superose 6 column relative to that of the starting VLP. [c] Average values, calculated from UV/Vis spectroscopy; experimental error = 10%.

The number of attached POx chains in each case was determined by UV/Vis spectroscopy, taking advantage of the differential absorbance of VLP and polymer at 258 and 220 nm, respectively (Table 1 and the Supporting Informa-

tion). Denaturing gel electrophoresis showed bands corresponding to coat proteins conjugated to zero, one, two, three, and four POx chains (Figure 3c), with a distribution consistent with the total number of POx molecules attached per capsid measured by the UV/Vis absorbance assay.

Of the random copolymers, **P3** contained approximately 2.5 alkyne moieties per polymer chain whereas **P4** had 5 reactive groups. In each case, the number of alkyne groups presented by the attached chains ($298 \times 2.5 = 745$ for **P3**, and $144 \times 5 = 720$ for **P4**) is in good agreement with the total number of azides presented on the capsid surface (720). Therefore, a highly efficient click reaction cascade apparently occurs, in which the formation of an initial triazole is followed by the rapid intramolecular CuAAC reaction of the remaining alkynes on the polymer chain with the azides on the scaffold to which it is attached. This cascade is expected since the overall particle concentration is low, and therefore a large increase in local azide/alkyne concentration occurs upon POx attachment.

Very few azide and alkyne groups should therefore remain on the Qβ conjugates of **P3** and **P4**. Indeed, subsequent CuAAC reaction of the Qβ-$\mathbf{P4_{144}}$ adduct with an excess of fluorescent azide (BODIPY-N_3) or alkyne (Alexa568 alkyne) gave rise to no attachment of the former and very little (< 3% of the possible 700–720 azides) of the latter. Therefore, either all of the POx alkynes were reacted or were inaccessible even to small molecules, while a small number of unreacted azides remained on the particle surface or within the capsid. SDS-PAGE analysis of denatured particles supported these findings (Figure 3c). Whereas the discrete 14 kDa subunit was observed for the VLP and its azide derivative **2**, no bands were observable for the Qβ-$\mathbf{P3_{298}}$ and Qβ-$\mathbf{P4_{144}}$ conjugates. This is consistent with extensive protein–polymer cross-linking, essentially converting each 180-subunit particle into a single hyperbranched core–shell molecule that cannot enter the electrophoresis gel.

The telechelic polymers **P1** and **P2** provided significantly different results. A greater number of the **P1** chains were grafted under identical CuAAC conditions (244 vs. 146 per particle). Although both polymers are of similar length and hydration properties,[18] the larger hydrodynamic diameter of the Qβ-$\mathbf{P1_{244}}$ conjugate (Table 1) is consistent with its higher grafting density (requiring more radial extension of the POx chains) and a stronger expected interaction of **P2** with the Qβ surface due to the amphiphilic character of PEtOx (**P2**).[19]

To determine the effects of the chemical modifications on the stability of secondary structure elements of the coat proteins, we performed far-UV circular dichroism (CD) studies. A scan of underivatized Qβ particles at 20°C (Figure 4a, black line) shows a strong CD signal at 218 nm, characteristic of proteins rich in β-sheet structures.[20] At 100°C, all secondary structure information was lost for the underivatized VLP (Figure 4a, gray line) because of thermally induced unfolding and precipitation of the coat protein. The thermal stability of the particles was thereby assessed by following the changes in the CD signal at 218 nm over a temperature gradient (Figure 4b). Unfolding occured within a small temperature range, indicating a fast, cooperative-like process (Figure 4b). Denaturation of the VLP was irreversible, as the cooling of denatured samples did not result in the reconstitution of secondary structure elements (Figure 4b). The thermal-transition experiment was used to calculate the melting temperature ($T_{M,app}$) for each modified VLP (Figures 4b,d,e, and the Supporting Information Figure S10), with the underivatized VLP showing a high value (85°C) expected for such a robust particle.

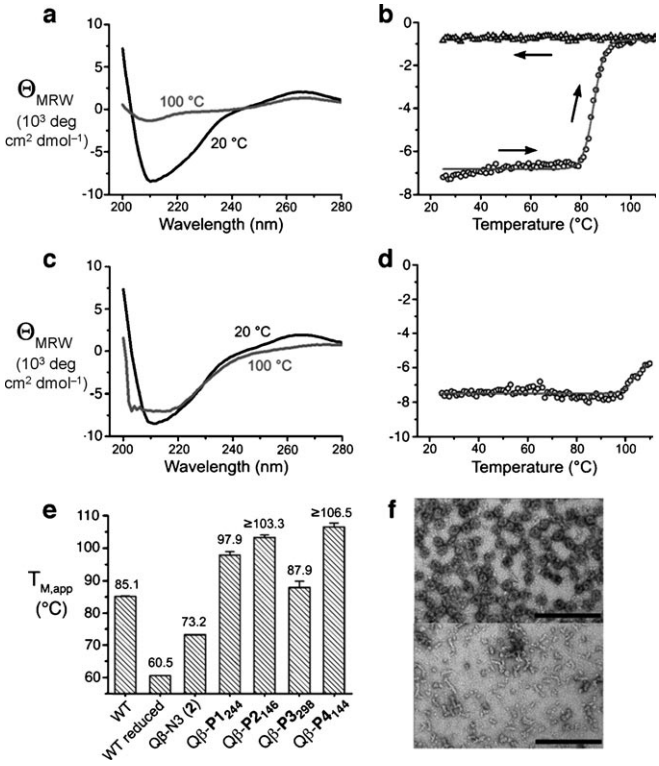

Figure 4. Stability assay of Qβ-POx conjugates. a–d) Far-UV CD spectroscopy: a) underivatized Qβ virus-like particles at 20°C (black) and after denaturation by heating at 100°C (gray). b) A cycle of gradual heating (○) and cooling (△) showing irreversible unfolding of the Qβ VLP protein, resulting in a loss of CD signal at 218 nm. A Boltzmann equation (solid line) was used to calculate the apparent melting temperature (see Supporting Information for details). c, d) Analogous to (a) and (b) for Qβ-$\mathbf{P2_{146}}$ particles. e) Calculated apparent melting temperatures ($T_{M,app}$) for each modified capsid measured and calculated as in (b). f) TEM images (magnification 92000×, scale bars 200 nm) of VLPs heated to 90°C for 10 min, intact Qβ-$\mathbf{P4_{144}}$ (top) and disrupted Qβ-N_3 (**2**) (bottom).

The importance of the characteristic inter-subunit disulfide linkages[4d] of Qβ was demonstrated by disulfide reduction and quenching of the resulting free cysteins by carboxymethylation. The resulting particles (Figure 4e, "WT reduced") were significantly less resistant to heating, showing a melting temperature of 61°C, similar to those of related bacteriophages without stabilizing disulfide bonds (MS2, 58-61°C; reduced PP7 virus, approximately 65°C).[4d,21] Interestingly, acetylation of surface lysines of particles containing intact disulfide bridges (Qβ azide **2**) also decreased the melting temperature (to 73°C; Figure 4e and the Supporting Information).

"POxylation" of the particles markedly enhanced their thermal stability, with $T_{M,app}$ values increasing to well above

that of the wild-type VLP (Figure 4e), as judged by the persistence of the native protein fold at high temperatures. Enhanced resistance to thermal denaturation of secondary structural elements was observed for the attachment of both end-functionalized (**P1**, **P2**) and multiply functionalized (**P4**, and to a lesser extent, **P3**) particles (Figure 4c–e). Because we could not carry out CD measurements at temperatures higher than 110 °C, the Boltzman fitting of these curves gave lower limits of the $T_{M,app}$ values for two cases that retained strong CD signals at 218 nm to the maximum temperature (Qβ-$\mathbf{P2}_{146}$ and Qβ-$\mathbf{P4}_{144}$). Notably, no protein precipitation was observed in the POx-functionalized samples after heating.

Information about tertiary and quaternary structure was provided by electron microscopy after heat treatment. Thermal denaturation at 90 °C of the Qβ-WT and Qβ-azide (**2**) particles as indicated by CD (Figure 4a,b) was accompanied by the appearance of large irregular assemblies in TEM images (Figure 4f and Figure S6 in the Supporting Information), consistent with loss of capsid integrity. The Qβ-$\mathbf{P1}_{244}$ and Qβ-$\mathbf{P2}_{146}$ conjugates appeared by CD spectroscopy to retain significant secondary structure at 90 °C (Figure 4c,d), but TEM revealed the particles to be disassembled into wormlike aggregates and spherical micelles, respectively (Figure S6). In contrast, the cross-linked Qβ-$\mathbf{P3}_{298}$ and Qβ-$\mathbf{P4}_{144}$ particles remained intact upon heating at 90 °C and 100 °C, respectively (Figure 4f and S7), demonstrating overall structural stability under extreme conditions. These data support the expectation that the noncovalent subunit–subunit interaction is the "weak link" in these particles, since heat-induced particle disassembly can occur without loss of the coat protein fold, as long as a telechelic POx polymer is attached to stabilize the latter (**P1** and **P2** adducts). Multiple-point polymer attachment and cross-linking is apparently necessary to preserve the packing of these proteins with each other to make the particle stable at elevated temperatures.

Poly(2-oxazoline)s are shown here to be compatible with Qβ VLP ligation in two different morphologies, giving rise either to end-attached polymer-decorated particles or to fully cross-linked core–shell structures. The latter are remarkably thermally stable, surviving temperatures in excess of 100 °C with little apparent loss of integrity. The CuAAC click reaction is efficient in joining densely packed azide and alkyne groups to each other, as we have observed in the context of adhesive materials.[22] The size of VLP–polymer constructs can be controlled by changing polymer chain length and attachment density. The ease, versatility, and functional group tolerance of the synthesis of poly(2-oxazoline) materials[23] and their click chemistry attachment to well-defined VLP scaffolds make this type of system of interest for both materials development and biological application.

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